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# Apiose and Mono-*O*-methyl Sugars as Minor Constituents of the Leaves of Deciduous Trees and Various Other Species

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1. Leaves of a number of species were hydrolysed with aqueous sulphuric acid and the resulting mixtures of sugars were fractionated by chromatography on activated charcoal. Paper chromatography of the fractions showed the presence in all the hydrolysates of minor constituents with $R_F$ values similar to or greater than those of the common hexoses and pentoses. 2. Two of these were identified as 2-*O*-methylxylose and 2-*O*-methylfucose. Estimates of the amounts present in whole leaves, and in fractions prepared from them, showed that they were associated with the hemicelluloses. 3. A third constituent was identified, by the formation of its di-isopropylidene derivative, as apiose. It also was associated chiefly with the hemicellulose fraction; none could be found in aqueous extracts from leaves of *Tilia vulgaris*, nor in aqueous extracts of *Zostera marina*, in which apiose is a major constituent of the water-insoluble polysaccharide. 4. A further constituent, after further purification by preparative paper chromatography, was tentatively identified, by gas–liquid chromatography of derivatives, as 3-*O*-methylgalactose, and was probably accompanied by small amounts of 4-*O*-methylgalactose. 5. These observations confirm the widespread occurrence of 2-*O*-methylxylose, 2-*O*-methylfucose and apiose, but 3-*O*-methylgalactose was hitherto known only in slippery-elm mucilage, and 4-*O*-methylgalactose in soil polysaccharides. Some experiments on the digestion of leaf hemicellulose fractions by snail crop-juice suggested that the mono-*O*-methyl sugars might confer resistance to enzymic degradation.

The polysaccharide component of humus has a sugar composition that varies very little from one soil to another (see Gupta, Sowden & Stobbe, 1963; Cheshire & Mundie, 1966). In addition to hexoses (glucose, galactose, mannose) and pentoses (arabinose, xylose, ribose), there are appreciable amounts of 6-deoxyhexoses (fucose, rhamnose) and much smaller amounts of partly methylated sugars (Duff, 1952*a*). The identification of some of these as 2-*O*-methylrhamnose, 4-*O*-methylgalactose (Duff, 1961), 2-*O*-methylxylose and 3-*O*-methylxylose (Bouhours & Cheshire, 1969) has raised more specifically the question of their origins. An answer to this question has an important bearing on a more general problem, namely whether the soil polysaccharide is mainly a microbial product, or alternatively consists mainly of those parts of plant polysaccharides that are more resistant to microbial degradation.

In 1959 a search was begun in this Department for partly methylated sugars in higher-plant material, particularly the leaves of deciduous trees, and soon confirmed the presence of 2-*O*-methylxylose and 2-*O*-methylfucose, then recently found by Andrews & Hough (1958) in leaves of plums and a number of other plants. By the use of a preliminary separation on activated charcoal, fractions rich in these sugars were obtained and rough estimates were made of the amounts present. Evidence was also obtained for the presence of apiose (Bacon, 1963) and for a substance having some of the properties of 4-*O*-methylgalactose.

After an interruption of several years it has been possible to return to this problem with the advantages of analytical methods developed in the interval, and we present here the evidence for the widespread occurrence of a number of minor sugar components in the water-insoluble fraction of higher plants.

## MATERIALS AND METHODS

*Plant material.* Whole plants of *Sinapis alba*, grown in solution culture, were provided by Dr P. C. DeKock (see DeKock, Hall & McDonald, 1960). Roots and leaves of mixed grass species were taken from a well-established lawn, freed from weeds by the use of selective herbicide treatments.

Leaves were taken from trees in the grounds of the Macaulay Institute, or its neighbourhood, some being collected in dry weather shortly after they had fallen (in late October 1959). Care was taken to exclude leaves that might have undergone microbial attack after reaching the ground.

A fresh sample of *Zostera marina* was very kindly collected under winter conditions (November 1962) from Loch Sween, Argyll, U.K., through the courtesy of Dr S. M. Marshall, F.R.S., of the Marine Biological Station, Millport, Isle of Cumbrae, U.K. Part was extracted as soon as it arrived in Aberdeen, cold and still wet with sea-water; the rest was stored in the frozen state. The identification of the material as *Z. marina* was confirmed by Professor T. G. Tutin.

Samples of the residual fibres of *Posidonia australis* were kindly provided by Miss E. M. Wollaston of the Department of Botany, University of Adelaide, Adelaide, S. Austral., Australia.

*Sugars.* Authentic samples were obtained as follows: 2-*O*-methyl-D-xylose and 2-*O*-methyl-L-fucose from Mrs E. E. Percival, and 3-*O*-methyl-D-galactose from Sir Edmund Hirst, F.R.S., Department of Chemistry, University of Edinburgh; di-isopropylideneapiose from Dr D. J. Bell, Department of Physiology, University of Edinburgh; 4-*O*-methyl-D-galactose from Professor J. K. N. Jones, F.R.S., Queen's University, Kingston, Ont., Canada. 2-*O*-Methyl-D-galactose and 6-*O*-methyl-D-galactose were samples in a collection made by the late Dr R. B. Duff.

*Chromatography and electrophoresis on paper.* The methods used were those described by Bacon (1959) or by Bouhours & Cheshire (1969), except that, during the course of the work, benzidine was named in the *Carcinogenic Substances Regulations* (1967), no. 879, and we replaced it as a spraying agent by *p*-anisidine (Hough, Jones & Wadman, 1950).

*Fractionation of hydrolysates.* The plant material, or a fraction derived from it, was hydrolysed by heating at 100°C in 0.5M-$H_2SO_4$ for 5h, with occasional shaking; typically 50g fresh wt. of leaf was so treated with 150–200ml of 0.5M-$H_2SO_4$. After filtration through sintered glass the hydrolysate was neutralized (internal indicator) with 2M-NaOH, and a small precipitate filtered off with the aid of a little Celite. The neutral solution was then run directly on to a column of 50g of Ultrasorb S.C. 120/240 (Carbo Norit Union Ltd., West Thurrock, Grays, Essex, U.K.) mixed with 50g of Celite no. 535 (Koch–Light Laboratories Ltd., Colnbrook, Bucks., U.K.), which had been packed in 5% (v/v) acetic acid and washed with 1 litre of water before use, and followed by water. A total of 500ml of effluent was collected. The sugars were then eluted from the column with 1 litre of 35% (v/v) ethanol (Hughes & Whelan, 1958). The eluate was evaporated to dryness, care being taken to maintain the pH between 5 and 7 during the process.

The residue was then dissolved in 5ml of water and put on a column of 40g of BDH Activated Charcoal (BDH Chemicals Ltd., Poole, Dorset, U.K.) and 40g of Celite no. 535. A gradient of aqueous ethanol made by adding 20% (v/v) ethanol to 1 litre of water (Bacon & Bell, 1953) was applied. The first 425ml of effluent was discarded. The second 425ml contained rhamnose, fucose, 2-*O*-methylxylose, 2-*O*-methylfucose and various sugars with $R_F$ values less than that of glucose, presumably oligosaccharides. When a determination of the methylated sugars was to be made this mixture was used without further fractionation.

The two *O*-methyl sugars may be separated from the fucose and rhamnose, and from each other, by gradient elution from a column of 4g of BDH Activated Charcoal and 4g of Celite no. 535 by using 40% (v/v) ethanol dropping into 200ml of water. For final purification it is necessary to remove substances of low $R_F$ values, which may be done by partition chromatography on Celite (Lemieux, Bishop & Pelletier, 1956).

*Determination of O-methyl sugars.* The fraction from the 40g–40g column referred to above was evaporated to dryness and 0.5ml of water was added. The solution was applied to a large sheet of paper, previously washed with distilled water, and divided as described by Bacon (1955) into five guide strips and four strips for analysis. Of the latter, two carried five 5$\mu$l spots of the test solution and the other two were left blank. After development with butan-1-ol–acetic acid–water (4:1:5, by vol.) overnight the paper was allowed to dry at room temperature and the guide strips were sprayed with benzidine–trichloroacetic acid reagent. Portions of the other strips corresponding to the positions of the methylated sugars were cut out and each pair was extracted with a measured volume of water, the minimum needed to provide duplicate samples for colorimetric analysis.

2-*O*-Methylxylose was determined by the method of Mejbaum (1939) on 3.5ml samples with a heating time of 40min (see Ashwell, 1957), and 2-*O*-methylfucose by the method of Dische (1955) with 1.0ml samples, with arabinose and rhamnose as standards respectively. Calibrations were made by using authentic samples of the two sugars: 100$\mu$g of 2-*O*-methyl-D-xylose was equivalent to 26$\mu$g of L-arabinose, and 100$\mu$g of 2-*O*-methyl-L-fucose to 86$\mu$g of D-rhamnose. The determinations on the blank strips gave consistent values of about 10$\mu$g/sheet for 6-deoxyhexose, and rather more variable values, usually in the range 10–20$\mu$g/sheet, for pentose. Each sheet carried one-tenth of the methylated sugars from the original leaf samples, amounts ranging from 50 to 250$\mu$g. On a few occasions rhamnose and fucose were also determined, and it was then possible to use larger quantities (0.5–1.0mg)/sheet.

*Infrared spectroscopy.* I.r. spectra of sugars in KBr discs, as thin films between NaCl plates or in chloroform solution were recorded on a Grubb–Parsons double-beam spectrophotometer, type 54, equipped with a NaCl prism.

## RESULTS

### 2-O-*Methyl*-D-*xylose and* 2-O-*methyl*-L-*fucose*

The conditions of acid hydrolysis used (5h at 100°C with 0.5M-sulphuric acid) were chosen originally for comparison with the hydrolysis of soil polysaccharide (Duff, 1961). Hemicelluloses are not completely hydrolysed under these conditions, but a second treatment of the insoluble residue did not yield significant additional amounts of the sugars being studied.

The acid solution was neutralized and subjected to chromatography on activated charcoal as described in the Materials and Methods section. Retention of the sugars on Ultrasorb charcoal was not always complete, but examination of the order of their elution from this column showed that the pentoses were first to emerge, so that any losses are not likely to have involved the partly methylated sugars.

The two sugars in question appeared on paper chromatograms sprayed with benzidine–trichloroacetic acid or *p*-anisidine hydrochloride reagents as a pink spot with $R_F$ 0.48 and a yellow spot with $R_F$ 0.55 in butan-1-ol–acetic acid–water (4:1:5, by vol.); the $R_F$ value of rhamnose is taken as 0.37. Careful examination of the eluates from BDH Activated Charcoal did not suggest any heterogeneity, and samples isolated from a fractionated hydrolysate of fallen beech leaves, by using a final separation by partition chromatography on Celite (Lemieux *et al.* 1956; see Bacon, 1959), crystallized at once when seeded with authentic specimens. Their identity was confirmed by i.r. spectra. A preparation of 2-*O*-methylxylose from mixed fractions originating in five species also crystallized readily.

No other sugars were seen with $R_F$ values greater than that of rhamnose in butanol solvents. The colorimetric measurements reported below, which were made on eluates from paper chromatograms, therefore probably refer in each case to a single sugar. Because of uncertainties about the completeness of hydrolysis and the possible destruction of sugar by the acidic conditions the values quoted cannot be taken as precise indications of the amounts present in combination in the plants, but seem sufficiently reproducible to give a picture of their distribution.

*Hydrolysis of whole plant material.* Hydrolyses were made of a variety of materials including fresh roots of mustard (*Sinapis alba*) and of mixed grass species, and fresh or autumn-shed leaves of mustard, of grasses and of several deciduous trees (Table 1): beech (*Fagus sylvatica* L.), horse chestnut (*Aesculus hippocastanum* L.), sweet chestnut (*Castanea sativa* Mill), sycamore (*Acer pseudoplatanus* L.), ash (*Fraxinus excelsior* L.) and a poplar (*Populus* sp.). In every case fucose and rhamnose were found, as well as the expected mixtures of hexoses and pentoses. This widespread occurrence of fucose is not usually recognized (see the discussion on p. 1540 after Roudier, 1960). Some measurements were made of the amounts of fucose and rhamnose liberated by a single period of hydrolysis, but cannot be taken as more than a rough indication of the totals present, because further amounts were liberated by a second hydrolysis of the residue; more rhamnose than fucose resisted the first hydrolysis. Typical figures for these 6-deoxy sugars after a single hydrolytic treatment are given in Table 2.

The amounts of the two mono-*O*-methyl sugars are given in Table 1. They were also found in the apiose-rich species *Posidonia australis* and *Zostera marina* (see below).

A few hydrolyses were made with less drastic conditions to test whether the methylated sugars were liberated preferentially. Hydrolysis of fresh beech leaves with 0.5% oxalic acid for 2h at 100°C yielded only one-fifth of the methylxylose and methylfucose released by the 0.5M-sulphuric acid for 5h.

Table 1. 2-O-*Methyl*-D-*xylose and* 2-O-*methyl*-L-*fucose in hydrolysates of leaves of deciduous trees*

| Species | Nature of sample | Sugar content (mg/100g sample) 2-*O*-Methylxylose | 2-*O*-Methylfucose |
|---|---|---|---|
| Ash (*Fraxinus excelsior*) | Leaves; autumn colour | 24 | 7.3 |
| | | 13 | 5.5 |
| Horse chestnut (*Aesculus hippocastanum*) | Leaves; autumn colour | 20 | 7.3 |
| Oak (*Quercus* sp.) | Leaves; green, September | 32 | 9.1 |
| Poplar (*Populus* sp.) | Leaves; autumn colour | 13 | 3.8 |
| Sweet chestnut (*Castanea sativa*) | Leaves; autumn colour | 26 | 9.5 |
| Sycamore (*Acer pseudoplatanus*) | Leaves; autumn colour | 21 | 5.7 |
| Beech (*Fagus sylvatica*) | Leaves; autumn | 18 | 6.4 |
| | Leaves; green, 26 May | 10 | 2.8 |
| | Leaves; green, 1 June | 10 | 2.2 |
| | Leaves; green, 20 June | 11 | 2.6 |

Table 2. *Distribution of fucose, rhamnose, 2-O-methylxylose and 2-O-methylfucose in fractions of beech* (Fagus sylvatica) *leaves*

Green leaves (50g; dry wt. 37%) picked in June 1960 and stored at −15°C for 5 months were extracted three times with 500ml of boiling 50% (v/v) ethanol, then four times with 2.5M-NaOH at 80°C. The whole of the 50%-(v/v)-ethanol extract and the final residue were hydrolysed for analysis of sugars. The NaOH extract was neutralized with acetic acid, dialysed against running tap water, concentrated to 110ml and poured into 550ml of ethanol; the precipitate was dehydrated with organic solvents and the whole hydrolysed for analysis. The recovery of dry matter in the three fractions was 84%.

| Fraction | Dry wt. (g) | Total sugar (mg/fraction) | | | |
|---|---|---|---|---|---|
| | | Fucose | Rhamnose | 2-*O*-Methylxylose | 2-*O*-Methylfucose |
| Soluble in 50% ethanol | 7.5 | 0 | 2.8 | 0 | 0 |
| Soluble in 2.5M-NaOH at 80°C | 3.6 | 4.1 | 8.8 | 2.3 | 0.9 |
| Insoluble residue | 4.6 | 0.8 | 1.0 | 0 | 0 |
| Hydrolysis of whole leaf | 17.8 | 5.8 | 7.2 | 4.8 | 1.1 |

*Differential extraction.* To confirm that the methylated sugars originated in polysaccharides, extractions of green beech leaves were made with 50% ethanol, water or EDTA (sodium salt) solution. Neither sugar could be detected in hydrolysates of the material extracted by 50% ethanol from young leaves (Table 2). Extraction with boiling water yielded some ethanol-precipitable material, more from young (May 1960) than from old leaves (October 1959). The former gave 5.8mg of methylxylose and 1.5mg of methylfucose from 100g dry wt. of leaves, compared with 26mg and 5.9mg by direct hydrolysis. Leaves of the same batch after exhaustive extraction with 0.5% EDTA (sodium salt) at pH6 at 90–95°C yielded a residue containing 23mg of methylxylose and 4.2mg of methylfucose/100g; the pectic material recovered from the extracts contained 1.5mg and 0.1mg of the sugars respectively. A number of crude pectic fractions were examined qualitatively; they contained very little of the methylated sugars. This is confirmed by the results in Table 3.

Extraction with 2.5M-sodium hydroxide at 80°C, which removed about 20% of the leaf dry weight, left a residue in which the methylated sugars could not be detected (Table 2).

Table 3 gives the results of a systematic fractionation of leaves of *Quercus petraea* by using the extraction procedure of Andrews & Hough (1958). The total recovery of the two sugars is not good, but it is evident that the greater part of both is located in the fraction dissolved by 2.5M-sodium hydroxide at 80°C.

All the above observations confirm the finding by Andrews & Hough (1958) that the methylated sugars are present in the hemicellulose fraction.

## *Apiose*

In early stages of this work, during the separation of the *O*-methyl sugars on paper chromatograms a spot was noticed, between fucose and rhamnose, giving an intense fluorescence in u.v. light after spraying with the benzidine–trichloroacetic acid reagent. This proved to be apiose (see Duff, 1965).

Since this sugar at that time was believed to occur only in certain plant glycosides, notably apiin, and in the polysaccharides of a few plant genera (*Posidonia, Zostera, Lemna*), an attempt was made to decide its location in *Tilia vulgaris* leaves, of which a large sample was available.

The identity of the sugar was first confirmed by separating it by preparative paper chromatography of fractions from Activated Charcoal–Celite columns, and converting it into the di-isopropylidene derivative by the method of Bell, Isherwood, Hardwick & Cahn (1954). Because the quantities present were small it was necessary to adapt their procedure (see Duff, 1965). After treatment with acetone and sulphuric acid the derivative, which is appreciably volatile (Bell, 1962), sublimed on to a 'cold finger' condenser, where it crystallized; it was then identified by its i.r. spectrum (D.M.S. no. 12900).

The quantity present may be judged from the following details of the procedure (extraction A). Fresh leaves (3kg; dry wt. 750g) were extracted four times with 10 litres of water at 90°C with Waring Blendor treatment. The residue was next extracted four times at room temperature with 1M-sodium hydroxide (10, 5, 5 and 5 litres), and finally with 2 litres of 2.5M-sodium hydroxide at 80°C. This extraction (for 3h) yielded about 3 litres of extract, which was clarified by passing it through a De Laval separator (size 1200; Alfa-Laval Co. Ltd., Brentford, Middx., U.K.), neutralized with acetic acid and dialysed against running tap water for 2 days. The material that was precipitated during dialysis was recovered by dehydration with ethanol (7.6g), and a further 10.9g was recovered by addition of 4vol. of ethanol to the supernatant. The two materials were com-

Table 3. *Distribution of* 2-O-*methylxylose and* 2-O-*methylfucose in fractions of oak* (Quercus petraea) *leaves*

Leaves (100g; dry wt. 40%), picked 6 September 1960, were extracted at once with boiling water four times; the extract (1440ml) was concentrated to 100ml, and 500ml of ethanol was added. The precipitate was dehydrated with organic solvents to yield 3.8g. The leaf residue was further extracted four times with M-NaOH at room temperature; the extract (1460ml) was neutralized with acetic acid, dialysed and concentrated to 80ml, and 5 vol. of ethanol was added, yielding a final precipitate of 3.7g. A similar extraction of the residue was made with 2.5M-NaOH at 80°C (2000ml), yielding 6.2g of crude polysaccharide. The final dried residue weighed 9.2g. Part of each fraction was hydrolysed as described in the Materials and Methods section and analysed for 2-*O*-methyl sugars.

| | Water-soluble | | Soluble in M-NaOH at room temp. | Soluble in 2.5M-NaOH at 80°C | Final residue | Totals | Whole leaf hydrolysate (11.10.60)* corrected to 40% dry matter |
|---|---|---|---|---|---|---|---|
| | 85% ethanol-soluble | 85% ethanol-insoluble | | | | | |
| Dry wt. of fraction in 100g fresh wt. of leaf (g) | Not measured | 3.6 | 3.5 | 5.9 | 8.8 | 21.8 | |
| Fraction as % of total dry matter in leaf | — | 9.0 | 8.7 | 14.6 | 21.9 | 54.2 | |
| 2-*O*-Methylxylose (mg/100g fresh wt. of leaf) | None | 1.0 | 0.7 | 8.2 | 1.2 | 11.1 | 14.4† |
| (mg/100g dry wt. of fraction) | None | 29 | 21 | 140 | 13 | 51 | 36 |
| (sugar in fraction as % of total sugar recovered) | None | 9 | 7 | 74 | 10 | [100] | |
| 2-*O*-Methylfucose (mg/100g fresh wt of leaf) | None | 0.34 | 0.26 | 1.8 | 0.16 | 2.5 | 4.1† |
| (mg/100g dry wt. of fraction) | None | 9.3 | 7.3 | 30 | 1.9 | 11 | 10 |
| (sugar in fraction as % of total sugar recovered) | None | 13 | 10 | 70 | 7 | [100] | |

* This hydrolysis was made on leaves that had dried to only 12% moisture content.

† By using these values as reference points the recovery of 2-*O*-methylxylose was 77% and that of 2-*O*-methylfucose was 61%.

bined, and 8.5g was hydrolysed in three equal batches. Each hydrolysate was purified on Ultrasorb and Activated Charcoal columns as described in the Materials and Methods section. The apiose-containing fractions from the second column were combined and separated on a sheet of Whatman Seed Test (1.6mm) paper. The three eluates were combined to give 13mg of a syrup in which a little rhamnose was the only other sugar detected by paper chromatography. Conversion into the isopropylidene derivative and sublimation yielded two fractions of crystalline material, totalling 9–10mg, which represented about 2mg of apiose/100g of dry matter. The final leaf residue from a similar extraction procedure (extraction B) yielded no apiose on hydrolysis.

Visual comparison of whole-leaf hydrolysates with standard amounts of apiose on paper chromatograms suggested that the sugar was present in smaller amounts than the two 2-*O*-methyl sugars. In extraction B 17.3g, 12.8g and 5.2g of polysaccharide were recovered from three successive hot 2.5M-sodium hydroxide extracts, and samples (10g each) of the first and second were hydrolysed in a search for 4-*O*-methylgalactose (see below). From the first sample the apiose-containing fractions were dissolved in 4.0ml of water, and portions were separated by preparative paper chromatography: (*a*) 90$\mu$l on Whatman 3MM paper yielded 6mg of eluate, and (*b*) a larger sample (0.9ml) was separated successively on Whatman Seed Test and Whatman 3MM papers and yielded 13mg. These yields are greater than those with extraction A, but paper chromatography showed the presence of fucose. Analysis by g.l.c. indicated that this sugar and apiose were present in comparable amounts and that (*b*) also contained a little rhamnose. Mannitol was used as an internal standard to determine the amounts present as alditol acetates, and indicated that (*b*) contained 5.6mg of apiose; this would correspond to 12mg/100g of dry matter, a value similar to those found for 2-*O*-methylfucose in various leaves (Tables 1 and 3).

Extracts of *Tilia* leaves made with water or 80% (v/v) ethanol were chromatographed on Whatman Seed Test paper. Various bands were eluted and hydrolysed in the hope of locating glycosides that contained apiose, but none was found.

A corresponding series of experiments was made

with the leaves of parsley (*Apium petroselinum*) and as expected apiose was found in hydrolysates of glycosides separated on Whatman Seed Test paper. Apiose was also present in the extracted residue, but in very small amounts.

Finally, fresh samples of *Zostera marina* were examined in the same way. Here there were only traces of apiose in the aqueous extract, but large amounts in the residue. It is noteworthy that both in *Zostera* and in *Posidonia* chromatographic evidence was obtained for the presence of methylxylose and methylfucose; the quantities present in a sample of *Posidonia* fibre were 10.8 and 3.1 mg/100 g respectively.

### 3-O-*Methylgalactose*

Systematic examination of fractions of leaf hydrolysates from charcoal columns showed that a number of other unidentified substances occurred regularly. They were recognized by the ease of their elution from charcoal and their $R_F$ values and colour reactions on paper chromatograms.

Paper chromatograms were occasionally run in aqueous phenol, particularly to establish the presence of galactose, which has a greater $R_F$ value than glucose in this solvent, but a smaller one in butanol-containing solvents. During the examination of a fraction from a leaf hydrolysate a spot was noticed with an $R_F$ value in phenol–water about 1.2 times that of rhamnose. 4-*O*-Methylgalactose was found to have a very similar $R_F$, so an attempt was made to prepare amounts large enough for its identification.

4-*O*-Methylgalactose has an $R_F$ value close to that of xylose in butanol–acetic acid–water, but is eluted later from charcoal columns (see Duff, 1961); after spraying with benzidine or *p*-anisidine reagents it has a yellow fluorescence in u.v. light, whereas xylose gives a pink colour with no fluorescence. Presumptive evidence for the presence of a mono-*O*-methylgalactose could therefore be obtained from the routine examinations of the eluates from 40 g columns of BDH Activated Charcoal. A spot with the expected characteristics began to appear in fractions immediately preceding the emergence of 2-*O*-methylxylose in hydrolysates of leaf fractions of beech, sweet chestnut and lime. Its properties were such that the most likely contaminants were xylose, from trailing on charcoal columns, and fucose, from trailing on subsequent paper chromatography. The quantities present were always very small, and repeated separations on thick paper or small charcoal columns involved inevitable losses.

Very small samples (1 or 2 mg) were obtained in this way from the hydrolysis of crude hemicellulose fractions from sweet-chestnut and lime leaves. I.r. spectra of these syrups were compatible with the presence of an *O*-methylgalactose, but would not distinguish the 3-*O*-methyl from the 4-*O*-methyl derivative. G.l.c. of the acetylated nitriles suggested that the substance from sweet chestnut was 3-*O*-methylgalactose and that from lime 4-*O*-methylgalactose.

A rather larger yield (11 mg) was obtained by hydrolysis of 100 g of sweet-chestnut leaf previously extracted exhaustively with hot 80% (v/v) ethanol. This fraction contained an unidentified impurity, but g.l.c. and electrophoresis in borate solution both indicated that 3-*O*-methylgalactose was present.

Further preparations were made from lime leaves. Hydrolysis of a polysaccharide sample (10 g) from extraction B (the second hot-2.5 M-sodium hydroxide extract; see above under 'Apiose') yielded 1.2 mg, which contained mainly 3-*O*-methylgalactose and showed some evidence for a small amount of 4-*O*-methylgalactose. Hydrolysis of 400 g of green lime leaves (dry wt. 138 g), collected in September, yielded 7.6 mg of material containing as major components 3-*O*-methylgalactose and an unidentified substance that was not xylose, arabinose, fucose, ribose, 2-*O*-methylgalactose or 6-*O*-methylgalactose; some 4-*O*-methylgalactose could be present in this sample also.

### *Fate of methylated sugars during enzymic hydrolyses of leaf polysaccharides*

Duff (1961) suggested that the presence of methylated sugars might hinder the biological degradation of polysaccharides in soil. A number of experiments were tnerefore carried out with snail digestive juice and water-insoluble preparations from leaves.

As expected, the snail juice liberated hexoses and pentoses from the water-insoluble residue of mustard roots. Some polysaccharide went into solution, and when precipitated by acetone and hydrolysed showed most of the sugars known to be present in the starting material, including methylxylose. In a similar experiment with milled EDTA-extracted green beech leaves the soluble sugars were examined more thoroughly, and fucose and rhamnose were identified in addition to the usual hexoses and pentoses. The polysaccharide solubilized by the enzymic action contained some methylxylose.

The snail digestive juice was tested on the four polysaccharide fractions from *Quercus petraea* (see Table 3). Hexoses and pentoses were released from all, but the water-soluble fraction yielded mainly arabinose, and the residue hardly any pentose at all. Rhamnose was liberated only from the fraction extracted with hot sodium hydroxide, so this

Table 4. *Distribution of fucose, rhamnose, 2-O-methylxylose and 2-O-methylfucose in fractions resulting from the action of snail digestive juice on a crude hemicellulose preparation from oak* (Quercus petraea) *leaves*

A 1 g portion of the 2.5 M-NaOH-soluble polysaccharide from leaves of *Quercus petraea* (see Table 3) was suspended in 9 ml of water and heated at 80°C with additions totalling 1.5 ml of 0.12 M-NaOH. Then 0.12 M-HCl was added to neutralize the suspension, 0.5 ml of M-sodium acetate buffer, pH 5.0, water to 15.5 ml and finally 2.0 ml of snail crop juice. After 7 days at 37–38°C the whole incubation mixture was heated at 95°C for 15 min and then centrifuged. The precipitate was washed twice with dilute buffer. Ethanol (5 vol.) was added to the combined supernatant and washed (23 ml). The resulting precipitate was washed by reprecipitation and dehydrated with organic solvents; the yield was 0.29 g. It and the water-insoluble fraction were hydrolysed before analysis, but the ethanol-soluble fraction was applied directly to an Activated Charcoal–Celite column.

| | Total sugar (mg/fraction) | | | |
|---|---|---|---|---|
| Fraction | Fucose | Rhamnose | 2-*O*-Methylxylose | 2-*O*-Methylfucose |
| Material soluble in 85% ethanol | 2.5 | 19.2 | Trace | Trace |
| Material insoluble in 85% ethanol | 1.1 | 2.1 | 0.21 | 0.18 |
| Water-insoluble residue | 1.4 | 1.1 | 0.44 | 0.07 |
| Substrate* | Not measured | Not measured | 1.4 | 0.30 |

* Values taken from analysis in Table 3.

fraction was incubated with snail juice in further experiments, from which the products of enzyme digestion were divided into water-insoluble residue, material precipitated from the incubation medium with 5 vol. of ethanol, and the ethanol-soluble fraction. Analyses were confined to the 6-deoxyhexoses and the 2-*O*-methyl sugars. No 2-*O*-methylxylose or 2-*O*-methylfucose was found in the free state, although large amounts of rhamnose and fucose were liberated. The distribution of these sugars in one experiment is given in Table 4. There was also a considerable liberation of pentose: 58 mg of arabinose and 86 mg of xylose in the experiment of Table 4. In a comparable experiment with 1.0 g of the hot-2.5 M-sodium hydroxide-soluble polysaccharide from lime leaves 4.4 mg of fucose, 21.6 mg of rhamnose and 156 mg of mixed pentose (arabinose plus xylose) were found in the free state. In an experiment resembling that of Table 4, except that one-tenth of the quantity of snail juice was used, smaller amounts of free fucose (1.1 mg) and rhamnose (1.6 mg) appeared in the ethanol-soluble fraction. After acid hydrolysis these increased to 2.3 mg and 10.9 mg respectively; a small amount of 2-*O*-methylxylose was detected in this hydrolysate, but no 2-*O*-methylfucose.

Thus, although there is extensive degradation of these crude hemicellulose preparations by the snail digestive juice, the 2-*O*-methyl sugars mostly remain in the ethanol-insoluble products.

## DISCUSSION

The findings reported here are the results of a search motivated by interest in the minor constituents of soil polysaccharides. For this reason we looked particularly for sugars with $R_F$ values greater than that of rhamnose. Duff (1952*a*) noted the presence of two spots, with $R_G$ values ($R_G = R_{\text{Tetra-}O\text{-methylglucose}}$; Hirst, Hough & Jones, 1949) 0.37 and 0.48 in butanol–acetic acid–water (rhamnose has $R_G$ 0.30). Duff (1961) later showed that both contained more than one sugar, and identified 2-*O*-methylrhamnose as a component of the $R_G$ 0.48 mixture. Bouhours & Cheshire (1969) showed that the $R_G$ 0.37 spot contained 2-*O*-methylxylose and 3-*O*-methylxylose and many unidentified minor components.

We could find no evidence for heterogeneity in the spots corresponding to 2-*O*-methylxylose and 2-*O*-methylfucose in leaf hydrolysates. The latter sugar, with $R_G$ 0.45, is a candidate for inclusion in Duff's (1952*a*) $R_G$ 0.48 spot, and its presence there has not been excluded. Plant material may therefore contribute a major component to each spot, but the minor components may well originate in micro-organisms.

Duff (1961) also found 4-*O*-methylgalactose in soil hydrolysates. This sugar is less readily noticed in chromatographic fractions of hydrolysates, because its $R_F$ value in most solvents is in the range of the common pentoses. It is present in the leaves of lime and sweet chestnut, but 3-*O*-methylgalactose, which has not so far been identified in soil, is more abundant in both species.

Cochrane, Gray & Arni (1969) found a monomethylgalactose that constituted 5–10% of certain hemicellulose fractions from the wood of sweet chestnut and wych elm. They tentatively identified it as 2-*O*-methylgalactose.

Apiose has not been seen in soil hydrolysates and was at one time thought to be restricted to a few genera of plants. The work of Duff & Knight (1963) (see Duff, 1965) indicated a wider distribution, and the present results suggest that it may be of universal occurrence, although in very small amounts.

The significance of the presence of all these sugars as minor constituents of hemicelluloses is not easy to explain, although the occurrence of small amounts of methylated bases and sugars in nucleic acids may be an analogous phenomenon (see Borek & Srinivasan, 1966; Trim & Parker, 1970). Plant nucleic acids tend to have higher percentages of methylated bases.

Duff (1961) referred to the possibility that methylation of a few sugar residues in a polysaccharide may block the action of carbohydrases, and the results of our experiments with snail digestive juice support this idea. Portions of polysaccharides containing these unusual sugars might thus tend to accumulate when plant material is being degraded in the soil, and so the soil polysaccharide fraction might be expected to become enriched in *O*-methyl sugars. In fact, the percentages estimated by Duff (1952*a*), namely 0.9% each of his $R_G$ 0.37 and $R_G$ 0.48 sugars in the polysaccharide–humic acid complex from a mineral soil, and by Bouhours & Cheshire (1969), namely 0.4% of *O*-methylxyloses in peat, are actually less than the values we find in whole leaves, and considerably less than those for crude hemicellulose preparations (see Table 3).

The first reaction to the discovery of fucose (Duff, 1952*b*) and *O*-methyl sugars in the soil polysaccharide fraction was a strengthening of the opinion that it is mainly microbial in origin (Whistler & Kirby, 1956; Duff, 1961). The present investigation, the work of Bouhours & Cheshire (1969) and the recent discovery in this Department that more than half the uronic acid present is galacturonic acid (C. M. Mundie, personal communication) all point to the possibility that partly degraded plant material may survive for long periods in the soil and must be taken into account when considering the contribution of polysaccharides to the preservation of soil structure.

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